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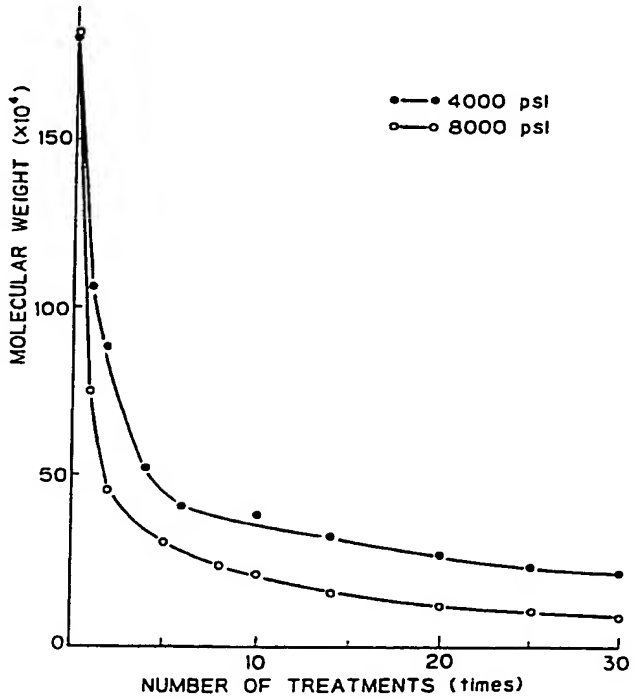
(54) Title: PROCESS OF PRODUCTION OF LOW-MOLECULAR WEIGHT HYALURONIC ACID

(57) Abstract

A process for producing hyaluronic acid having a viscosity average molecular weight of 500,000 or less by mechanically degradating a high molecular hyaluronic acid solution by a shear treatment.



DEGRADATION OF HA BY MANTON GAULIN

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DESCRIPTION

Process of Production of Low-Molecular Weight Hyaluronic Acid

TECHNICAL FIELD

The present invention relates to a process for the production of low-molecular weight hyaluronic acid (hereinafter referred to as "HA"). More specifically, it relates to a process for the production of a low molecular hyaluronic acid which is stable against heat and which can be industrially produced in a large production scale.

BACKGROUND ART

HA is produced by extracts from rooster combs, umbilical cord, skin, synovial fluid, and other biological tissues or by the fermentation method using bacteria of genus Streptococcus and is used for cosmetics and pharmaceuticals. Heretofore, in this field, high molecular weight HA has been mainly used due to the fact that the higher the molecular weight and viscosity of the HA, the more noticeable the effect. However, high-molecular weight HA has many problems in the application thereof. For example, high molecular weight HA is poor in heat stability and is required to storage at low temperatures. Furthermore, HA cannot be heat sterilized. This has limited the development of applications of HA.

On the other hand, it has been found that low-molecular HA differs from the high-molecular HA in that it easily dissolves in water and is low in viscosity. Accordingly, there are such advantageous effect that cosmetics including it do not give an unpleasant feeling such as stickiness to the skin or stinginess. Thus, the use of low-molecular HA as a cosmetic material has been expected. Further, low molecular HA has a healing effect on wounds and, therefore, is considered applicable as, for example, eye

drops, skin ointments, anti-adhesion agents.

It has been heretofore known to obtain low-molecular HA by treating high-molecular weight HA (for example, an average molecular weight of 700,000 or more) by hyaluronidase and also heat, strong acid, strong alkali, and other chemical methods (Japanese Unexamined Patent Publication (Kokai) No. 62-79790 and Japanese Unexamined Patent Publication (Kokai) No. 63-57602).

However, the conventional processes for production of low-molecular HA have suffered from difficulties in control of the degree of decomposition and have given various molecular weight of hyaluronic acids, in addition to the HA of the desired molecular weight, and, as a result, an extremely broad distribution of molecular weights of the HA (in other words, an uneven molecular weight of the hyaluronic acid) is produced. Further, it is difficult to remove the hyaluronidase, acid, or alkali added for the treatment, and also there are problems such as a low yield. In addition, research on the heat stability of the resultant low-molecular weight HA has not yet been done.

DISCLOSURE OF INVENTION

Accordingly, the objects of the present invention are to eliminate the above-mentioned disadvantages of the prior art and to provide a process for producing a low-molecular weight hyaluronic acid having a viscosity average molecular weight of 500,000 or less and having a narrow molecular weight distribution at a high production yield.

Other objects and advantages of the present invention will be apparent from the following description.

In accordance with the present invention, there is provided a process for producing hyaluronic acid having a viscosity average molecular weight of 500,000 or less, preferably 15,000 to 500,000, by mechanically

degradating (or depolymerizing) a high-molecular weight hyaluronic acid solution by a shear treatment.

BRIEF DESCRIPTION OF DRAWINGS

The present invention will be better understood from the description set forth below with reference to the accompanying drawings, in which:

Figure 1 shows the relation between the times of treatment of high-molecular weight HA by a MANTONGAULIN and the resultant molecular weight;

Fig. 2 shows the results of a heat stability test at 40°C and 60°C of an aqueous low-molecular weight HA solution;

Fig. 3 shows the results of a heat stability test at 40°C and 60°C of an aqueous high-molecular weight HA solution; and

Fig. 4 shows the gel filtration chromatogram of low-molecular weight HA by Sephacryl S-1000.

BEST MODE FOR CARRYING OUT THE INVENTION

According to the present invention, the low-molecular weight HA having an average molecular weight of 500,000 or less, preferably 15,000 to 500,000 can be obtained by mechanically treating high-molecular weight HA (average molecular weight of 700,000 or more, preferably 700,000 to 2,500,000) by a shear treatment. The resultant low-molecular weight HA has an extremely narrow distribution of molecular weights (i.e., gives a substantially uniform molecular weight) and further is stable with respect to heat.

The molecular weight distribution of the low-molecular weight HA produced according to the present invention is 1.7 or less using the Mw/Mn ratio as the index of the molecular weight distribution (Mw: weight average molecular weight, Mn: number average molecular weight).

In the present invention, the mechanical shear treatment is used for the method of degradating or depolymerizing high-molecular weight HA to a low-

molecular weight. This shear treatment under a high shear rate gives HA of an extremely narrow molecular weight distribution, a substantially uniform molecular weight, compared with treatment by conventional chemical methods (e.g., ultraviolet rays, electron beams, free radicals, alkali, acid, heat, enzymes). Further, there is no need for removal of, for example, hyaluronidase, acid, or alkali, which is considered difficult in the past, and also the yield is high and a large scale industrial production can be carried out.

The shear treatment under a high shearing force or a high shear rate according to the present invention means the share rate is 10^5 dyne/cm^2 or more.

The shear treatment by a high shearing force according to the present invention can be effected by an emulsifier capable of applying a stronger shear than the mixers usually used for emulsification (e.g., homomixers, Disper mixers, propeller agitators). Examples of such emulsifiers are a MANTONGAULIN, French press, microfluidizer, colloid mill.

In the treatment using a shearing force of the present invention, the higher the pressure applied to the HA solution or the smaller the sectional area of the small spaces for passage, the higher the shear force created. In general, however, since the flow rate of the solution passing through the small spaces is large, the reduction in the molecular weight by a single treatment is insufficient and, therefore, it is desirable that the same operation be repeated or that multiple stage apparatuses is used to reduce the molecular weight to the desired level.

The starting HA used in the present invention may be any commercially available fowl tumor derived HA, microorganism desired HA, or relatively uniform high molecular weight HA obtained by cultivating HA producing bacteria under strictly controlled conditions and harvesting the same, so long as it is an HA of a high

molecular weight or a relatively high molecular weight (for example, an average molecular weight of 700,000 or more, preferably 700,000 to 2,500,000).

The production of HA according to the present invention is performed in an aqueous solution state. The concentration of HA in the solution is not particularly limited, but usually up to 2% by weight is preferable. When the concentration is more than 2% by weight, the viscosity becomes large and handling becomes inconvenient, which is not preferable from the viewpoint of commercialization.

In the present invention, the temperature of the solution during the shear treatment of HA has a large effect on the effect of the degradation to a low molecular weight. However, when a temperature is too high, a browning reaction and the like may occur and, therefore, the temperature is usually selected to be from room temperature to 100°C.

The starting HA is dissolved in water or a suitable saline solution and when degradated to the desired average molecular weight, separation and purification are performed by the usual, known operations such as organic solvent fractionation, salting out, dialysis, ultrafiltration, whereby it is possible to obtain HA having a narrow distribution of molecular weights with any average molecular weight below 500,000 and stable with respect to heat.

The low-molecular weight HA according to the present invention obtained in this way has in itself a wound curing effect, and, therefore, can be applied to eye drops, skin ointment, anti-adhesion agents, and other pharmaceuticals and cosmetics.

The HA obtained according to the present invention has an extremely narrow distribution of molecular weight and a substantially uniform molecular weight. Further, it is superior in heat stability. Further, there is no need for removal of the hyaluronidase, acid, or alkali,

which is considered difficult in the conventional chemical methods, the yield is high, and a large scale industrial production can be carried out.

EXAMPLES

The present invention will now be further illustrated by, but is by no means limited to, the following Examples.

Example 1

HA having a viscosity average molecular weight of 1,800,000 was dissolved in water to prepare two liters of a 0.3% by weight aqueous solution. This aqueous solution was degradated into low molecular form by a MANTONGAULIN of APV Gaulin Co. The pressure was set to 8,000 psi for 5 minutes and 4,000 psi for 10 minutes and the treatment was repeated under the same conditions to find the relationship between the number of treatments and the molecular weight. The results are shown in Fig. 1. The molecular weight was calculated by measuring the intrinsic viscosity [h] and the formula of Laurent $h = 36 \times Mw^{0.78} \times 10^{-5}$ (wherein Mw is a weight average molecular weight). As a result, for example, with 30 treatments at 8,000 psi, it was possible to produce HA having any average molecular weight up to a molecular weight of 500,000 or less.

Example 2

A heat stability test was performed at 40°C and 60°C for a 0.1% by weight aqueous solution of the low molecular HA obtained in Example 1. The results are shown in Fig. 1. HA's having an average molecular weight of 97,000, 155,000, 276,000, and 485,000 were stable for over six months. HA with an average molecular weight of over 1,800,000 (i.e., Comparative Example, Fig. 3), however, clearly fell in molecular weight.

Example 3

The Sephacryl S-1000 chromatograms of HA having an average molecular weight of 155,000 obtained in

Example 1 and HA having an average molecular weight of 162,000 obtained by heat decomposition (Comparative Example) are shown in Fig. 4.

As clear from the figures, the HA obtained by degradation to low molecular form by the shear treatment had a narrower distribution of molecular weight and more uniform molecular weight compared with HA obtained by heat treatment.

CLAIMS

1. A process for producing hyaluronic acid having a viscosity average molecular weight of 500,000 or less by mechanically degradating a high-molecular weight hyaluronic acid solution by a shear treatment.

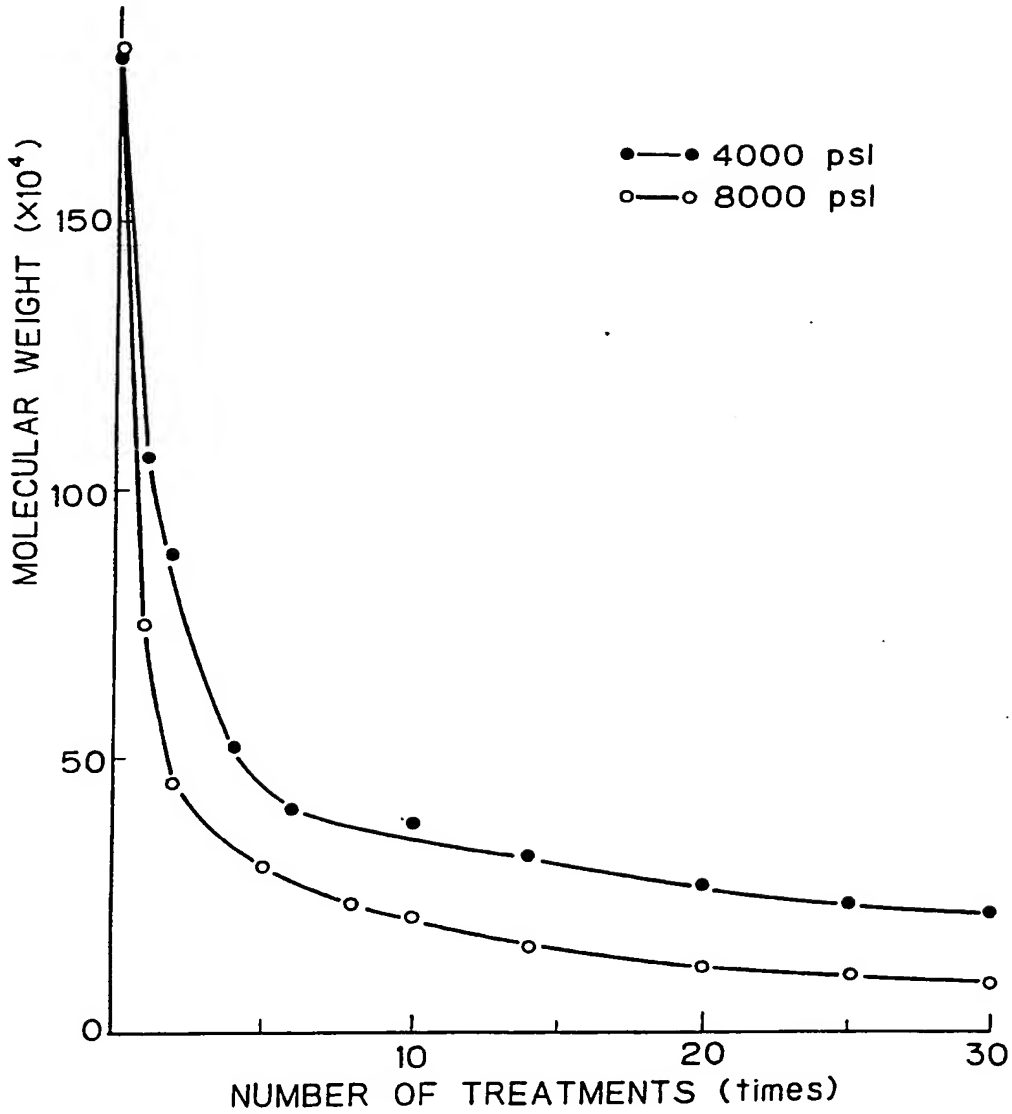
2. A process as claimed in claim 1, wherein the shear treatment is carried out at a shear rate of 10^5 dyne/cm^2 or more.

3. A process as claimed in claim 1, wherein the viscosity average molecular weight of the high-molecular weight hyaluronic acid to be treated is 700,000 or more.

4. A process as claimed in claim 1, wherein the viscosity average molecular weight of the resultant hyaluronic acid is 15,000 to 500,000.

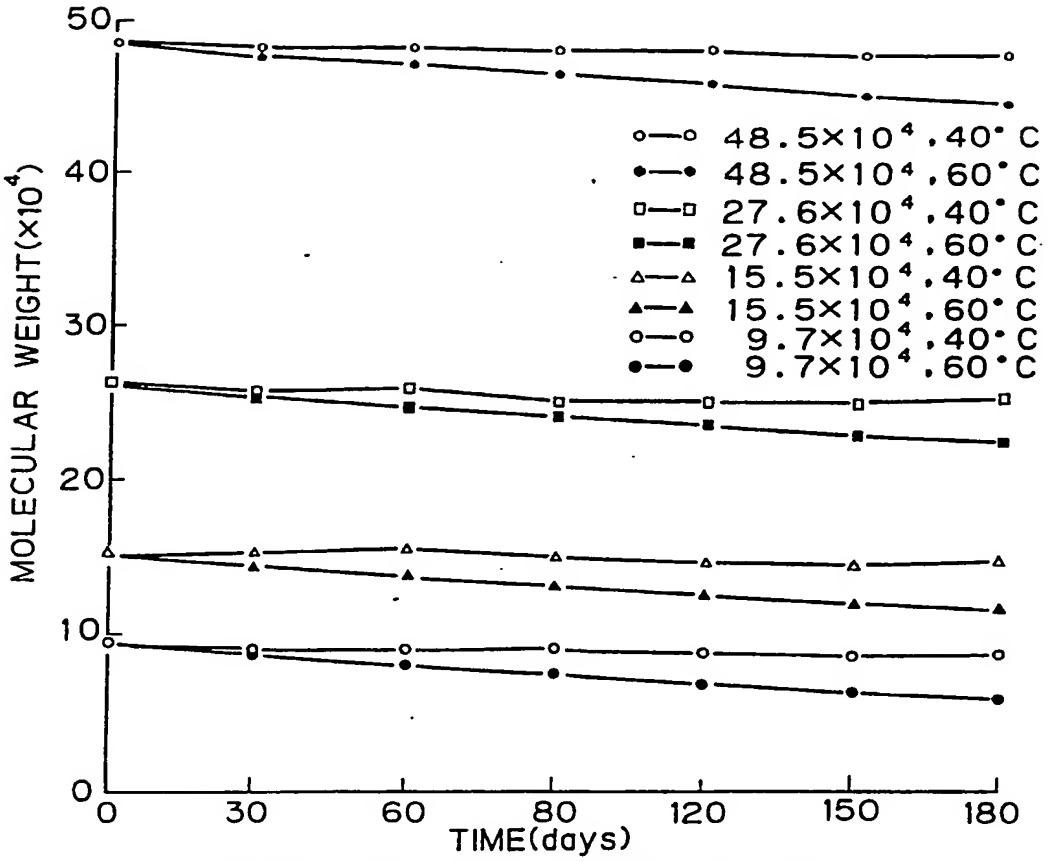
5. A process as claimed in claim 1, wherein the molecular weight distribution of the resultant hyaluronic acid is 1.7 or less in terms of the Mw/Mn ratio.

Fig. 1



DEGRADATION OF HA BY MANTON GAULIN

Fig. 2



RESULT OF HEAT STABILITY TEST AT 40°C AND 60°C OF AQUEOUS LOW-MOLECULAR WEIGHT HA SOLUTION

Fig. 3

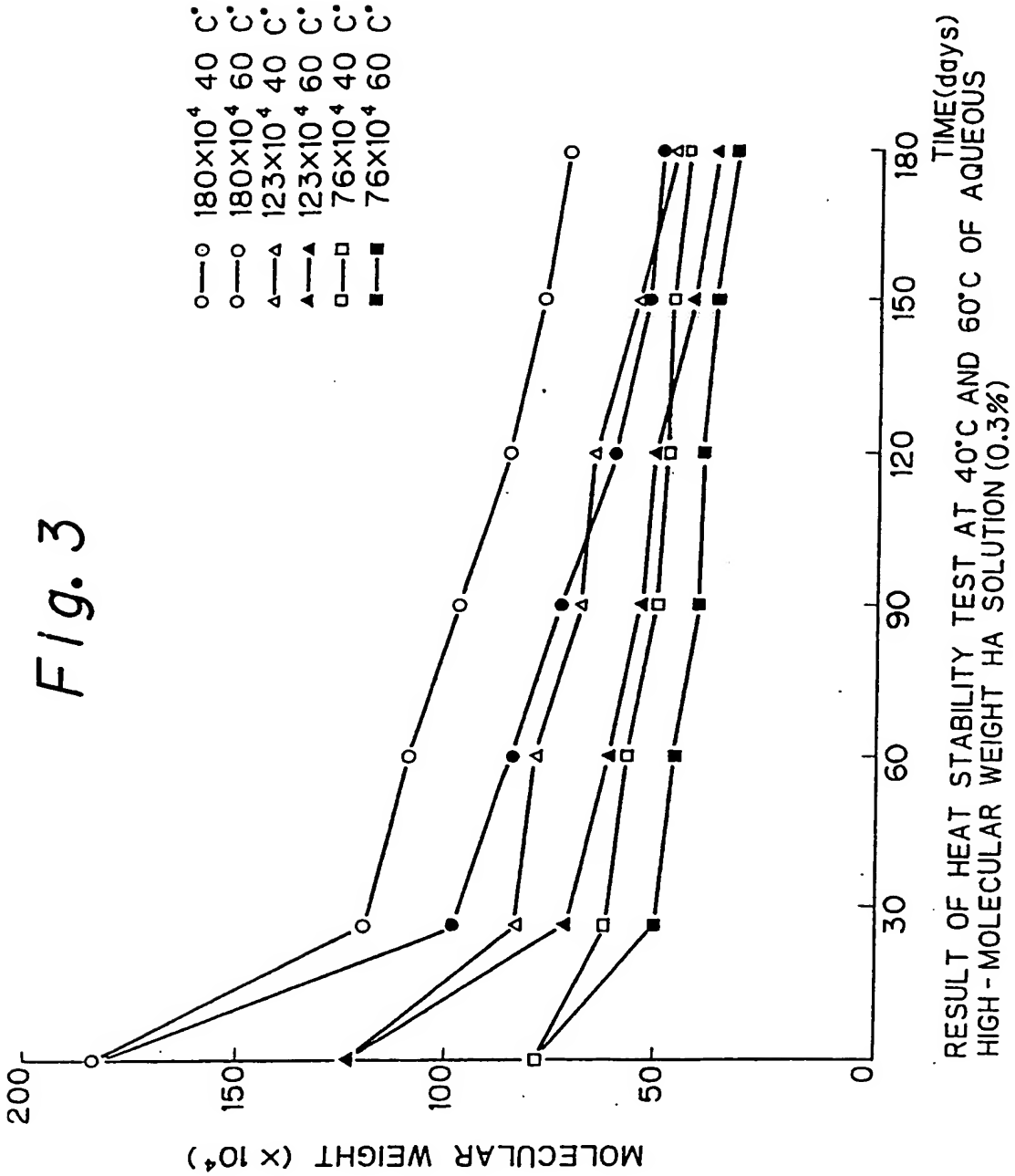
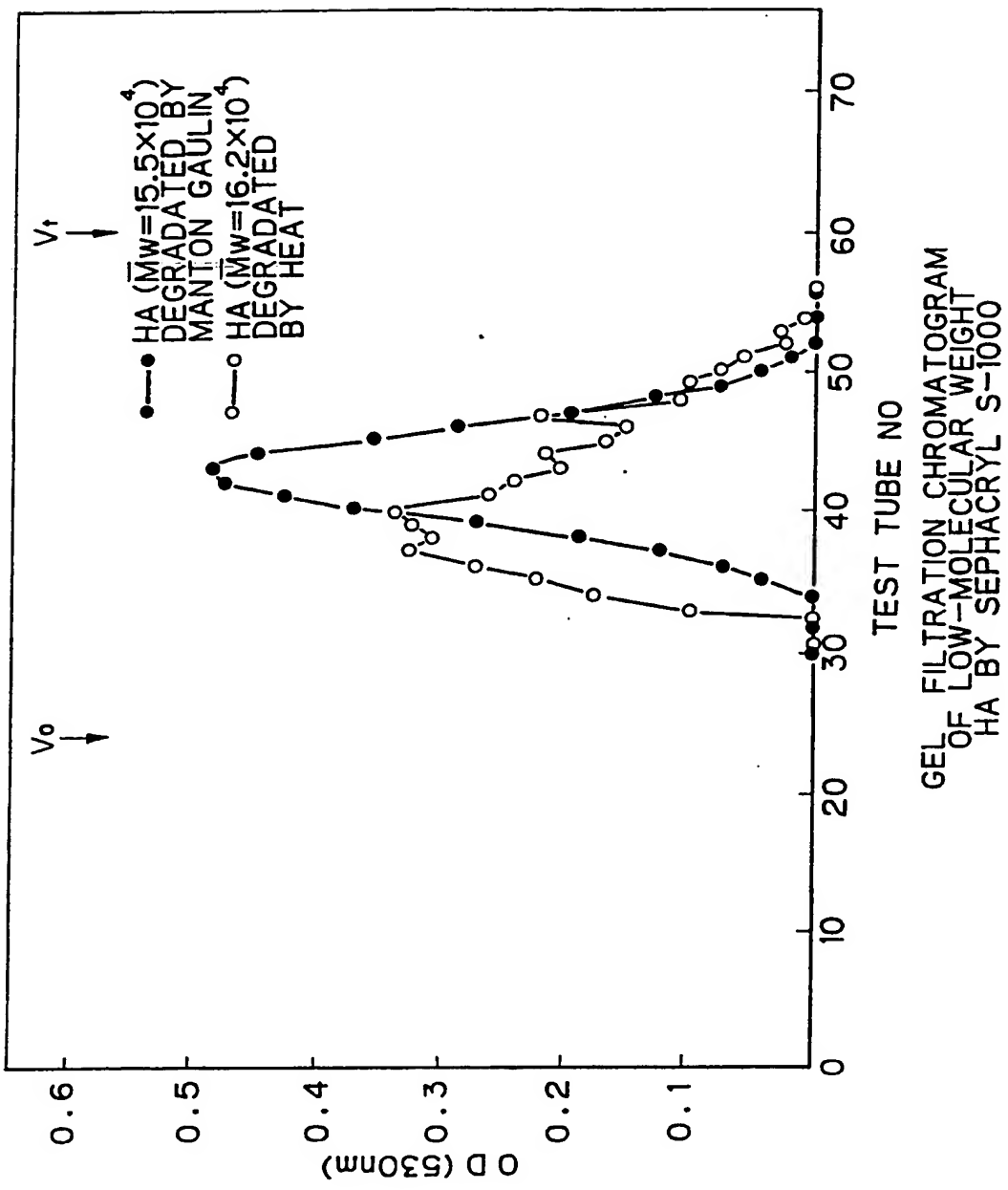

RESULT OF HEAT STABILITY TEST AT 40°C AND 60°C OF AQUEOUS HIGH-MOLECULAR WEIGHT HA SOLUTION (0.3%)
MOLECULAR WEIGHT (× 10⁴)
TIME(days)
200
150
100
50
0
30
60
90
120
150
180
180×10⁴ 40 C°
180×10⁴ 60 C°
123×10⁴ 40 C°
123×10⁴ 60 C°
76×10⁴ 40 C°
76×10⁴ 60 C°

Fig. 4



GEL FILTRATION CHROMATOGRAM OF LOW-MOLECULAR WEIGHT HA BY SEPHACRYL S-1000

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP 90/01168

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C08B37/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C08B

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	FR,A,2521569 (KAKEN PHARMACEUTICAL CO.) 19 August 1983 see page 1, lines 30 - 36 see page 2, lines 21 - 23 see page 3, lines 14 - 31 see page 4, lines 4 - 5, 19 - 21	1-4
Y	EP,A,216453 (FIDIA S.P.A.) 01 April 1987 see page 41, lines 11 - 22 see page 42, lines 5 - 15	1-4
A	EP,A,138572 (FIDIA S.P.A.) 24 April 1985 see page 2, lines 15 - 17 see page 3, lines 15 - 19 see page 4, lines 20 - 22 see page 5, line 23 - page 6, line 2 see page 15, lines 1 - 3	1

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 NOVEMBER 1990	20. 12. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	R.J. Eernisse

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,239335 (INTERNATIONAL PHARMACEUTICAL PRODUCTS, INC.) 30 September 1987 see column 2, lines 19 - 22 see column 2, line 45 - column 3, line 6 see column 4, lines 9 - 12	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

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